AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows:

1. Please add the following nine paragraphs taken from Columns 11-12 of U.S. Pat. No. 6,093,302, which was incorporated by reference into the above-identified application at the bottom of page 1, to page 9 before the heading "Immunoassays" and after the paragraph ending with "electrode" on the bottom of page 8:

The present invention provides methods for the preparation and use of a substrate having one or a plurality of chemical species in selected regions. The present invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could be readily applied to the preparation of other polymers, as well as to the preparation of sequences of nucleic acids. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either alpha-, beta-, or omega- amino acids, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides. In another preferred embodiment, the present invention is used for the synthesis of oligonucleotides and/or DNA.

The present invention is directed to placing molecules, selected generally from monomers, linker molecules and pre-formed molecules, including, in particular, nucleic acids, at a specific location on a substrate. The present invention is more particularly directed to the synthesis of polymers at a specific location on a substrate, and in particular polypeptides, by means of a solid phase polymerization technique, which generally involves the electrochemical removal of a protecting group from a molecule provided on a substrate that is proximate at least one electrode. The present invention is also particularly directed to the synthesis of oligonucleotides and/or DNA at selected locations on a substrate, by means of the disclosed solid phase polymerization technique.

Electrochemical reagents capable of electrochemically removing protecting groups from chemical functional groups on the molecule are generated at selected electrodes by applying a sufficient electrical potential to the selected electrodes. Removal of a protecting group, or

"deprotection," in accordance with the invention, occurs at selected molecules when a chemical reagent generated by the electrode acts to deprotect or remove, for example, an acid or base labile protecting group from the selected molecules.

In one embodiment of the present invention, a terminal end of a monomer nucleotide, or linker molecule (i.e., a molecule which "links," for example, a monomer or nucleotide to a substrate) is provided with at least one reactive functional group, which is protected with a protecting group removable by an electrochemically generated reagent. The protecting group(s) is exposed to reagents electrochemically generated at the electrode and removed from the monomer, nucleotide or linker molecule in a first selected region to expose a reactive functional group. The substrate is then contacted with a first monomer or pre-formed molecule, which bonds with the exposed functional group(s). This first monomer or pre-formed molecule may also bear at least one protected chemical functional group removable by an electrochemically generated reagent.

The monomers or pre-formed molecules can then be deprotected in the same manner to yield a second set of reactive chemical functional groups. A second monomer or pre-formed molecule, which may also bear at least one protecting group removable by an electrochemically generated reagent, is subsequently brought into contact with the substrate to bond with the second set of exposed functional groups. Any unreacted functional groups can optionally be capped at any point during the synthesis process. The deprotection and bonding steps can be repeated sequentially at this site on the substrate until polymers or oligonucleotides of a desired sequence and length are obtained.

In another embodiment of the present invention, the substrate having one or more molecules bearing at least one protected chemical functional group bonded thereto is proximate an array of electrodes, which array is in contact with a buffering or scavenging solution. Following application of an electric potential to selected electrodes in the array sufficient to generate electrochemical reagents capable of deprotecting the protected chemical functional groups, molecules proximate the selected electrodes are deprotected to expose reactive functional groups, thereby preparing them for bonding. A monomer solution or a solution of pre-formed molecules, such as proteins, nucleic acids, polysaccharides, and porphyrins, is then contacted

with the substrate surface and the monomers or pre-formed molecules bond with the deprotected chemical functional groups.

Another sufficient potential is subsequently applied to select electrodes in the array to deprotect at least one chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group. A second monomer or pre-formed molecule having at least one protected chemical functional group is subsequently bonded to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule. The selective deprotection and bonding steps can be repeated sequentially until polymers or oligonucleotides of a desired sequence and length are obtained. The selective deprotection step is repeated by applying another potential sufficient to effect deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule. The subsequent bonding of an additional monomer or pre-formed molecule to the deprotected chemical functional group(s) occurs until at least two separate polymers or oligonucleotides of desired length are formed on the substrate.

Preferred embodiments of the present invention use a buffering or scavenging solution in contact with each electrode, which is buffered towards the electrochemically generated reagents, in particular, towards protons and/or hydroxyl ions, and that actively prevents chemical cross-talk caused by diffusion of the electrochemically generated ions from one electrode to another electrode in an array. For example, when an electrode exposed to an aqueous or partially aqueous media is biased to a sufficiently positive (or negative) potential, protons (or hydroxyl ions) are produced as products of water hydrolysis. Protons, for example, are useful for removing electrochemical protecting groups from several molecules useful in combinatorial synthesis, for example, peptides, nucleic acids, and polysaccharides.

In order to produce separate and pure polymers, it is desirable to keep these protons (or hydroxyl ions) confined to the area immediately proximate the selected electrode(s) in order to minimize, and, if possible to eliminate, chemical cross-talk between nearby electrodes in an array. The spatial extent of excursion of electrochemically generated reagents can be actively controlled by the use of a buffering or scavenging solution that reacts with the reagents that move away from the selected electrodes, thus preventing these reagents from reacting at a nearby electrode.

2. Please add the following paragraph taken from Columns 19-20 of U.S. Pat. No. 6,093,302, which was incorporated by reference into the above-identified application at the bottom of page 1, to page 9 before the heading "Immunoassays", paragraph, and after the last paragraph identified above in amendment request #1 and ending with "at a nearby electrode":

The molecules of the invention, i.e., the monomers, linker molecules and pre-formed molecules, can be attached directly to the substrate or can be attached to a layer or membrane of separating material that overlays the substrate. Materials that can form a layer or membrane overlaying the substrate, such that molecules can be bound there for modification by electrochemically generated reagents, include: controlled porosity glass (CPG); generic polymers, such as, teflons, nylons, polycarbonates, polystyrenes, polyacylates, polycyanoacrylates, polyvinyl alcohols, polyamides, polyimides, polysiloxanes, polysilicones, polynitriles, polyelectrolytes, hydrogels, epoxy polymers, melamines, urethanes and copolymers and mixtures of these and other polymers; biologically derived polymers, such as, polysaccharides, polyhyaluric acids, celluloses, and chitons; ceramics, such as, alumina, metal oxides, clays, and zeolites; surfactants; thiols; self-assembled monolayers; porous carbon; and fullerine materials. The membrane can be coated onto the substrate by spin coating, dip coating or manual application, or any other art acceptable form of coating.

3. Please replace the **Abstract** with the following amended paragraph:

There is disclosed a process and an array for assaying for binding of target molecules to capture molecules on microarray devices, wherein the ~~mircoarray~~ microarray devices contain electrodes. Specifically, there is disclosed a binding (including nucleotide hybridization) process to detect binding on a microarray wherein the microarray contains electronically addressable electrode devices. There is further disclosed an enzymatically catalyzed oxidation/reduction reaction to take place within a "virtual flask" region of a microarray wherein the reaction is detected by current changes detected on the addressable electrode.

4. Please replace the first full paragraph on page 2 with the following amended paragraph:

With regard to placing fully-formed oligonucleotides at specific locations, various micro-spotting techniques using computer-controlled plotters or even ink-jet printers have been

developed to spot oligonucleotides at defined locations. One ~~techniques~~ technique loads glass fibers having multiple capillaries drilled through ~~then~~ them with different oligonucleotides loaded into each capillary tube. Microarray chips, often simply glass microscope slides, are then stamped out much like a rubber stamp on each sheet of paper of glass slide. It is also possible to use "spotting" techniques to build oligonucleotides in situ. Essentially, this involves "spotting" relevant single nucleotides at the exact location or region on a slide (preferably a glass slide) where a particular sequence of oligonucleotide is to be built. Therefore, irrespective of whether or not fully-formed oligonucleotides or single nucleotides are added for in situ synthesis, spotting techniques involve the precise placement of materials at specific sites or regions using automated techniques.

5. Please replace the fourth full paragraph on page 7 with the following amended paragraph:

Figure 14 shows a 3D plot for oligonucleotide hybridization electrochemical detection. Specifically, rabbit and Kras oligonucleotide sequences were in situ synthesized on an electrode containing microarray device. The chip was set up in an alternating electrode-counter electrode format having a checkerboard pattern of sites having a Kras (or rabbit) oligonucleotide capture probe sequence surrounded diagonally by counter electrodes without oligonucleotides synthesized thereon. Target Kras sample (Operon) was treated to form single-stranded DNA (Operon) and biotinylated with Kras complement (Operon) according to manufacturers instructions. Streptavidin conjugated with HRP (Sigma) was added to the biotinylated Kras sequence complement to form a target complex or complementary Kras affinity-bound to HRP. The target Kras sample complexed with HRP was added to the chip and each electrode was measured for current (amps). These data are shown in Figure 14 in the top panel in a 3D ~~plat~~ plot and in the bottom panel showing a positive signal in a checkerboard for Kras oligonucleotide capture probes and the bottom panel showing no signal for rabbit sequence captures probes.

6. Please replace the first paragraph under the heading "Electrode-Based Microarrays" on page 8 with the following amended paragraph:

Electrode-based microarrays can be made with various oligomers attached to predefined regions, wherein each predefined region is defined by the presence of an addressable electrode. An addressable electrode is one where it can be electronically accessed to create a current or voltage. Electrode-based microarrays further and often comprise a porous matrix layer that holds the capture molecules and provides a three dimensional virtual flask (cylindrical in the case of a circular electrode). In a preferred embodiment, the porous matrix layer is a membrane, wherein the membrane material is selected from the group consisting of polyvinyl alcohol, polyvinyl acetate, polyvinyl alcohol, tricellulose acetate, polyurethane, agarose, controlled porosity glass with a PTFE resin, and combinations thereof. In each case, the microarray, contains a plurality (~~on~~ only in rare cases only one) of capture molecules. In the most common form of microarray, the capture molecules are oligonucleotides than can bind to complementary sequence regions (or nearly complementary sequence regions depending upon the hybridization conditions) of DNA or mRNA from the target samples. The challenge next becomes how one can detect this binding event or hybridization event. In terms of marketed ~~products,~~ products that are generally made by spotting or ink-jet printing oligonucleotides onto planar, non-porous surfaces such as glass slides, there are sample labeling kits commercially available that cause the sample nucleic acid to become labeled with a fluorescent dye. Often it is a fluorescent dye sold under the trademarks of Texas Red®, or Cy® Dyes Cy3 and Cy5. The microarray is "read" through a common fluorometer arrangement with either microscopic magnification or imaging stitching and looking for fluorescence at the known locations where the capture molecule was spotted or synthesized. This common technique of fluorescent detection of microarrays using a standard fluorometer configuration with a microarray is the detection method universally used. However, there are optics issues, difficulty in labeling with fluorescent dyes, occasional high background problems and most importantly, extremely high costs associated with fluorescent microscopic equipment. Therefore, there is a need to detect molecular binding on microarrays using lower cost equipment. The present inventive method uses electrochemical reagents generated locally within a porous reaction layer or membrane to only locally provide current or voltage to a nearby electrode, whose current or voltage signal can be detected at the nearby electrode and not "cross-talk" onto neighboring electrodes.

7. Please replace the paragraph under the heading "Immunoassays" on page 9 with the following amended paragraph:

Immunoassays are based generally upon antibody binding to another molecule, generally a protein sugar or glycoprotein. The problems of immunoassays are generally detection of this binding event. The sandwich based immunoassays are based upon the fact that one antibody has already been attached to the surface of the chip (the capture antibody; most often a monoclonal). The analyte is then bound to the antibody and a second antibody (usually polyclonal) is added for use as a reporter group. The second reporter antibody will generally contain a fluorophore or have an enzyme covalently attached. Alternatively, the reporter antibody may contain a biotin molecule. To this biotin molecule, a streptavidin-enzyme conjugate can be an attached. Therefore, the inventive process can be constructed with immunoassays, even sandwich-type immunoassays by providing for the oxidation/reduction enzyme to be attached to a complex formed when binding to a capture molecule (i.e., first antibody) occurs. The latter assay ~~formats~~ format allows a host of generic assay format to be designed without performing the grueling task of providing (synthesizing) analyte-based individual antibody-enzyme conjugates. Examples of immunoassays in a sandwich configuration are shown in Figures 1 and 2.

8. Please replace the first full paragraph on page 10 with the following amended paragraph:

β-Galactosidase reaction cleaves penultimate β-galactose residues from oligosaccharides or from glycosyl derivatives. The reaction scheme is shown in Figure 3. The substrate used was X-Gal, which is an indolyl derivative of β–galactopyranoside. Biotinylated β–galactosidase was purchased for studies. The reactions were carried out at pH 7.0 in PBS buffer, 0.01 M. A given quantity of X-Gal was dissolved in DMF (very soluble) so that when it was added to the aqueous buffer, a substrate concentration of about 0.1 mM was achieved (saturation). The X-Gal/DMF solution was added while vigorously vortexing the aqueous phase because of the limited solubility of the X-Gal in water. If the DMF/X-Gal solution is added without vigorous vortexing, the X-Gal would precipitate from solution. The instability of this solution requires that a fresh solution needs to be prepared daily.

9. Please replace the paragraph beginning on the bottom of page 11 and ending on the top of page 12 with the following amended paragraph:

The redox curve for the peroxidase reaction was monitored by a single 100 micron diameter electrode on a bare (*i.e.*, no porous membrane or capture molecules synthesized thereon) electrode-containing microarray device and is shown in Figure 10. The cyclic voltamogram like results indicate at a negative potential, the amperometric difference with and without HRP enzyme was substantial. Taking these data, an amperometric experiment was performed over a period of time (Figure 11). It should be noted that the current flow leveled off after a period of time. Thus, the best time to begin these studies is after about 2 minutes (Figure 11).

10. Please replace the last paragraph on page 13 with the following amended paragraph:

The ability of microarray devices to have synthesized many different capture molecules at different known locations allows for multiple analyte detection on a single chip. In each case the sample ~~or samples~~ (or samples) to be investigated is (are) labeled with an oxidation/reduction enzyme through standard conjugation means. Multiple samples can be pooled so that all of the targets to be investigated can be found from a single pooled sample. In one experiment samples of AGP, ricin and rabbit mRNA samples were pooled and investigated on a single chip using laccase as the oxidation/reduction enzyme. Only those known locations having the appropriate capture molecules detected target even though the group of targets were either protein or nucleic acids. Based upon multiple microarray investigations, the limits of detection were found to be 5 pg/ml for AGP and 300 pg/ml for ricin that translates to 2.5fM in a volume of 0.5 ml. Moreover, the dynamic detection range spanned four logs.

11. Please replace the paragraph on page 3 beginning with "Preferrably, the array" and ending on page 4 with the following amended paragraph:

Preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is generated by a technique selected from the group consisting of *in situ* synthesis with electrochemical techniques, spotting the capture molecules, ink-jet printing

the capture molecules, and *in situ* synthesis through photolighography techniques. Most preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is formed by *in situ* synthesis with electrochemical techniques. Preferably, the oxidation/reduction enzyme is selected from the group consisting of laccase, horseradish peroxidase, β-galactosidase, glucose oxidase, alkaline phosphatase, dehydrogenases, and combinations thereof. Preferably, the oxidation/reduction enzyme is attached to the target molecule(s) through an antibody and anti-idiotype antibody combination or through a biotin and streptavidin (or avidin) binding combination. Preferably, the array having a plurality of electrodes further comprises a porous reaction layer covering the electrodes, wherein the porous reaction layer has a thickness of from about 0.1 microns to about 10 microns and whereby the porous reaction layer functions to block diffusion of oxidation/reduction activity products such that there is little lateral signal being picked up at an adjacent electrode. Most preferably, the porous reaction layer is made from a polymeric material selected from the group consisting of polyvinyl alcohol, polyvinyl acetate, ~~dextrane~~dextran, epoxy-based polymers, tricellulose acetate, polyurethane, agarose, controlled porosity glass with a PTFE resin, and combinations thereof. Preferably, the capture molecule is a molecule from the class of molecules selected from the group consisting of oligonucleotides, polypeptides, antibodies, glycosylated polypeptides, polysaccharides, and mixed molecules having monomers from a plurality of the foregoing molecules. Similarly, the target molecule is one likely to bind to at least one of a plurality of capture molecules. Most preferably, a target molecule is from a class of molecules selected from the group consisting of DNA, RNA, single-stranded DNA, ribosomal RNA, mitochondrial DNA, cellular receptors (*i.e.*, glycosylated or non-glycosylated membrane-bound proteins), polypeptides, glycosylated polypeptides, antibodies, cellular antigenic determinants, organic molecules, metal ions, salt anions and cations, and combinations thereof.